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## Human Immunodeficiency Virus-Infected Individuals Contain Provirus in Small Numbers of Peripheral Mononuclear Cells and at Low Copy Numbers

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**In human immunodeficiency virus (HIV)-infected individuals, the proportion of circulating mononuclear cells (PBMCs) which carry HIV provirus and the number of HIV proviral sequences per infected PBMC have been matters for conjecture. Using a double polymerase chain reaction which allows the detection of single molecules of provirus and a method of quantifying the provirus molecules, we have measured provirus frequencies in infected individuals down to a level of one molecule per  $10^6$  PBMCs. As a general rule, only a small proportion of PBMCs contain provirus (median value of samples from 12 patients, one per 8,000 cells), and most if not all of the infected cells carry a single provirus molecule. The frequency of provirus-carrying cells correlated positively both with the progression of the disease and with the success with which virus could be isolated from the same patients by cocultivation methods. Of seven asymptomatic (Centers for Disease Control stage II) patients, all but one contained one provirus molecule per 6,000 to 80,000 cells; of five Centers for Disease Control stage IV patients, all but one contained one provirus molecule per 700 to 3,300 cells. When considered in conjunction with estimates of the frequency of PBMCs that express viral RNA, our results suggest that either (i) the majority of provirus-containing cells are monocytes or (ii) most provirus-containing lymphocytes are transcriptionally inactive. We also present nucleotide sequence data derived directly from provirus present *in vivo* which we show is not marred by the *in vitro* selection of potential virus variants or by errors introduced by Taq polymerase. We argue from these data that, of the provirus present in infected individuals, the proportion which is defective is not high in the regions sequenced.**

Human immunodeficiency virus (HIV) causes a chronic and eventually fatal disease in humans. The time course of the disease is slow and involves the persistence of virus infection in the face of a specific antiviral response by all arms of the immune system. On primary infection there is an initial viremia, which is followed rapidly by antibody production and viral clearance (1, 5, 7). However, protective immunity is not established, and signs of active infection reappear after a variable period of time. Progression to the acquired immunodeficiency syndrome (AIDS) correlates with the reappearance of circulating viral proteins (7, 23) and pathological evidence of extensive viral infiltration in many parts of the body, such as brain, gut, spleen, lymph nodes, and lungs (24, 26, 30, 31).

The mechanism by which HIV persists is unclear. It has been proposed that the extensive sequence variation seen in the *env* gene allows the virus to evade the immune response (8, 32). Another possibility is that the virus evades the immune system by virtue of a low level of gene expression in some of the cells that it infects. Cells of the monocyte-macrophage lineage are more resistant to the cytolytic effect of HIV infection *in vitro* than are T4 lymphocytes (6). Cells of the former type are considered by many investigators to constitute the main reservoir of HIV infection and to be the means by which viral infection is disseminated to multiple sites in the body (11, 21, 28). In support of this view, HIV can readily be isolated from macrophages. On the other hand, there is also evidence that virus expression in periph-

eral blood mononuclear cells (PBMCs) from AIDS patients can be low or absent for long periods of time (35), and nonexpressing T4 lymphocytes can be identified after *in vitro* infection (4, 12).

Little is known about the distribution of proviral DNA in cells of the peripheral circulation. Neither the number of infected cells, the number of copies of provirus per cell, nor the cell type which contributes most to the progression of the disease are known with any certainty. Clearly, it is of fundamental importance to establish these basic parameters of the disease. Previous attempts to measure the prevalence of provirus and the frequency of infected cells in PBMCs from infected individuals have been only moderately successful. Shaw et al. (30) detected HIV-specific sequences in only 1 of 22 samples of DNA extracted from PBMCs of AIDS and AIDS-related complex patients. The Southern blotting method used in this case allowed the detection of provirus molecules at a frequency of one per 200 or fewer cells, suggesting that the prevalence of provirus-carrying cells is usually lower than this. Ulrich et al. (34) titrated PBMCs from asymptomatic HIV-infected patients before attempts at isolation and found that the number of PBMCs required to obtain a positive result varied widely between individuals. One patient yielded a positive culture from only 100 cells, while most required  $10^4$  to  $10^5$  cells, and two remained negative even when  $10^6$  cells were used. Harper et al. (9) used *in situ* hybridization to measure the number of HIV-expressing cells in PBMCs from a similar group of symptomatic patients. Positive cells were seen in only half of the samples tested, and in these samples their frequency was

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again very low (one cell in  $10^4$  to  $10^5$ ). While these estimates are in good agreement, each poses problems of interpretation (see Discussion). In a number of published studies, the polymerase chain reaction (PCR) has been used to detect HIV proviral DNA sequences in PBMCs from seropositive patients. A striking feature of the results obtained so far is the scarcity or apparent absence of viral DNA in a proportion of patients (3, 10, 13, 22). None of these reports includes an assessment of the sensitivity of the method in terms of the minimum number of HIV molecules required to produce a positive signal. However, the results do suggest that the proportion of infected cells within the PBMC population may be extremely low in many cases.

Here we describe a modified PCR method with a sensitivity sufficient to detect a single molecule of target DNA. After a first PCR amplification with a pair of HIV-specific primers, a small proportion of the product was used as the template for a second round of PCR amplification using a second set of primers nested within the first pair. By combining this double PCR method with a limit dilution approach, both the proportion of infected cells and the number of molecules of HIV provirus per cell can be accurately estimated. Results obtained with PBMCs from 12 HIV-positive hemophiliacs showed a wide range in the proportion of provirus-carrying cells, with a median value of one provirus in about 8,000 cells; each infected cell carried about one copy of the provirus. The approach described here also provides a way to analyze sequence heterogeneity within populations of HIV provirus which avoids the complications that arise because of the lack of fidelity of the Taq polymerase (33) and other artifacts associated with amplification.

## MATERIALS AND METHODS

**Materials.** Seropositive blood samples were donated by members of a cohort of HIV-1-infected hemophiliacs. Samples from 16 seronegative patients from a low-risk category were used as negative controls. T4/T8-cell ratios were measured by fluorescence-activated cell sorting analysis, and T4 cells and monocytes were estimated by fluorescence microscopy. PBMCs were separated by centrifugation over Lymphopaque (Nyegaard) and washed twice with phosphate-buffered saline.

HIV primers (Fig. 1) were synthesized by the Oswel DNA Service, Department of Chemistry, University of Edinburgh, and were purified by high-performance liquid chromatography. Control primers corresponded to positions 9412 to 9430 (sense) and 9682 to 9664 (antisense) of the human alpha-1-antitrypsin gene (GenBank accession no. KO2212).

**Virus isolation.** The washed cells were suspended in RPMI (Gibco) supplemented with 20 mM L-glutamine; 10% heat-inactivated fetal calf serum; and 300 IU of penicillin, 0.3 mg of streptomycin, 120 mg of sodium bicarbonate, 10  $\mu$ g of Polybrene, 100 U of anti-human alpha interferon (ICN Pharmaceuticals Inc.), 150 U of recombinant interleukin-2 (Du Pont Co.), and 5 mg of phytohemagglutinin (Vector) per ml. A similar number of cells prepared from a seronegative donor in the same way 3 to 7 days previously was added on the first day and at 7-day intervals thereafter. Virus production was assayed at 3- to 4-day intervals for at least 30 days by using a HIV-1 p24-antigen capture enzyme-linked immunosorbent assay (Du Pont) according to the instructions of the manufacturer.

**DNA preparation.** The PBMCs were suspended in 400  $\mu$ l of lysis buffer (50 mM Tris hydrochloride [pH 8.0], 100 mM NaCl, 50 mM EDTA, 1% sodium-*n*-lauroylsarcosine, 100  $\mu$ g

### gag PRIMERS:

881:	5'GGTACATCAGGCCATATCACC;	sense; 1214
	(A)	HIV-MAL
	(A)	HIV-RF
882:	5'ACCGGCTCATACAGTCTC;	anti-sense; 1669
	(TA)	HIV-MAL
	(G)	HIV-PV22
	(A)	HIV-SF2
883:	5'GAGGAAGCTGCAGAATGGG;	sense; 1407
	(A)	HIV-ELI
	(C)	HIV-MAL
990:	5'GGTCCTTGTCTTATGTCCAGAATGCTG;	anti-sense; 1646
	(T)	HIV-CDC41
	(C)	HIV-ELI
	(C)	HIV-MAL

### pol PRIMERS:

001:	5'CATGGGTACCAGCACACAAAGG;	sense; 4149
004:	5'TCTACTTGTCCATGCATGGCTTC;	anti-sense; 4380
002:	5'GGAGGAATGAACAAGTAGATAAATTAGTCAG;	sense; 4175
	(G)	HIV-RF
003:	5'TCACTAGCCATTGCTCTCCAATT;	anti-sense; 4290

### env PRIMERS:

401:	5'GAGGATATAATCAGTTTATGG;	sense; 6539
	(G)	HIV-CDC42
404:	5'AATTCATGTGTACATTGTACTG;	anti-sense; 6976
402:	5'GATCAAAGCCTAAAGCCATG;	sense; 6560
	(A)	HIV-ELI
	(A)	HIV-MAL
	(A)	HIV-WMJ22
403:	5'CAATAATGTATGGGAATTGG;	anti-sense; 6876
	(G)	HIV-MN

FIG. 1. Primer sequences and coordinates. The coordinates listed are from HIV HXB2 (GenBank accession number K03455). Mismatches between the primer sequences and 10 (*gag*) or 9 (*pol* and *env*) published HIV-1 sequences are indicated in parentheses.

of proteinase K per ml) and incubated at 65°C for 2 h. The DNA was mechanically sheared by pipetting it up and down 30 times through a 1-ml Eppendorf tip, extracted twice with equal volumes of phenol and once with chloroform, and precipitated with 1 ml of ethanol. RNA was not routinely removed during preparation of DNA, since it is a minor component (20 to 25%) of the total nucleic acids in mononuclear cells in vivo (14, 16) and would not significantly affect the quantification of HIV provirus. After centrifugation, the nucleic acid pellet was suspended in TE buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) and estimated spectrophotometrically. All dilutions of DNA for PCR were made in a solution of 100  $\mu$ g of sheared herring sperm DNA per ml.

**Dilution of cells and DNA minipreparations.** The concentration of PBMCs was measured with a hemocytometer, and cells were diluted appropriately in phosphate-buffered saline. DNA was prepared essentially as described above, except that the lysis buffer was supplemented with 2  $\mu$ g of carrier herring sperm DNA. The pellet centrifuged out of ethanol was dried, 20  $\mu$ l of distilled water was added, and the tube was heated to 95°C for 7 min. The entire sample was then used as template for a single PCR reaction in a total volume of 70 to 100  $\mu$ l. By extracting both carrier DNA and known amounts of cells in the same way, the overall efficiency of the method was found to be 60 to 75%.

**Double PCR method.** The template DNA was present in 25 to 100  $\mu$ l of 67 mM Tris hydrochloride (pH 8.8), 16.7 mM ammonium sulfate, 6.7 mM  $MgCl_2$ , 10 mM 2-mercaptoethanol, 6.7  $\mu$ M EDTA, 33  $\mu$ M each of dATP, dCTP, dGTP, and TTP, 170  $\mu$ g of bovine serum albumin per ml, 10%

dimethylsulfoxide, 0.5  $\mu$ M of each of the outer nested primers, and 0.015 U of Taq polymerase (Cetus) per  $\mu$ l. Twenty-five heat cycles were used, with each consisting of 0.6 min at 94°C, 0.7 min at 50°C, and 3 min at 68°C. Two microliters of the reaction mixture was then transferred to a second tube containing the same medium as before but with the inner pair of nested primers, and a further 25 heat cycles were carried out with the same program.

The product of the second reaction (20  $\mu$ l) was analyzed by means of a 3% low-melting-point agarose gel containing ethidium bromide. The pTZ18R *Hae*III markers have lengths of 767, 458/434, 281/267, 174, 142, 137, 102, and 80 bp. The extreme sensitivity of the PCR method means that contamination of samples by even very small amounts of extraneous target sequence may generate false-positive reactions (18). This is particularly true of the more-sensitive double PCR method. By keeping stock solutions and samples and assembling the reactions in a room from which normal manipulation of cloned HIV sequences was excluded, potential contamination was avoided; of 40 negative-control herring sperm DNA samples, all were negative. Negative controls were carried through all experiments. To act as a further negative control, all batches of samples for DNA preparation included a negative-control sample of cells from a seronegative donor.

In order to confirm that human DNA itself does not generate positive results in the double PCR, DNA samples were prepared from 36 low-risk seronegative donors. Each was assayed in the double PCR with at least two of the three sets of primers (*env*, *gag*, or *pol*). None of the samples was positive in any assay, but all were positive after a single control PCR (25 cycles) carried out with the alpha-1-anti-trypsin primers.

**Length variation and nucleotide sequence analysis of double PCR products.** The double PCR products obtained with pBH10.R3 and lambda HAT3 templates were resolved by electrophoresis on a 5% denaturing polyacrylamide gel. The second PCR reaction was performed with inner primers end labeled by using polynucleotide kinase and [ $^{32}$ P]ATP (Amersham Corp.; 300 Ci/mmol). The gel was dried and exposed to X-ray film for 1 h.

To increase the amount of product available, a triple PCR was performed with the *gag* primers. The second set of cycles was carried out with one inner (883) and one outer (882) primer, and the third set was carried out with both inner primers as usual. In this way, each single initial template molecule yielded about 1  $\mu$ g of product. The product DNA was purified by treating the reaction mixture with Gene-Clean (Bio 101, Inc.) and eluting with TE buffer. Approximately 500 ng of DNA was mixed with 5 ng of  $^{32}$ P-labeled primer 883 in TE buffer, denatured at 95°C for 5 min, and annealed at 55°C for 15 min. Nucleotide sequencing was carried out by using the Sequenase-2 kit (United States Biochemicals) with [ $^{35}$ S]dATP according to the instructions of the manufacturer.

## RESULTS

The PCR depends critically on the hybridization of the primers to specific sequences in the target DNA, and it is therefore important that the primers should correspond to the most invariant regions of the genome. There is considerable sequence diversity between different isolates of HIV-1. For example, the overall identity between HIV-HXB2, a North American isolate, and HIV-MAL, from Zaire, is 87.9%. The *gag* and *pol* genes are more highly conserved

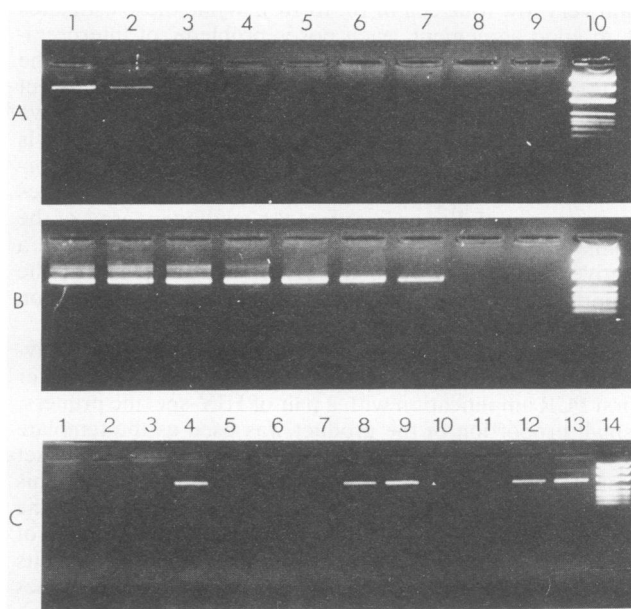


FIG. 2. Single and double PCR with serial dilutions of pBH10.R3 DNA. The *gag* primers shown in Fig. 1 were used. (A) Single PCR. (B) Double PCR. A sample from each single PCR was run on an agarose gel (A). Another sample was used to initiate the second PCR, and a sample of the double PCR product was also run on the same agarose gel (B). The quantities of template added to the first set of PCR reactions were as follows for the respective lanes: 1, 6.5 pg; 2, 650 fg; 3, 65 fg; 4, 6.5 fg; 5, 650 ag; 6, 65 ag; 7, 6.5 ag; 8, 0.65 ag. Lane 9 contains carrier herring sperm DNA alone, and lane 10 contains DNA markers (*Hae*III digest of pTZ18R). Extra bands of higher molecular weight were seen when relatively large amounts of template were transferred to the second reaction. Such DNA constitutes the linear accumulation of DNA copied from an inner primer-binding site to the end of DNA molecules bounded by the outer primers. (C) Samples, each nominally containing 6.5 ag, were simultaneously assayed in the double PCR. Lanes: 1, carrier DNA (negative control); 2 through 12, nominal 6.5-ag samples; 13, 65-ag sample; 14, markers.

than the *env* gene (89.3 and 90.6%, respectively, versus 85%). The nested sets of *gag*, *pol*, and *env* sequences that were used in the work described here were chosen to be as highly conserved as possible (Fig. 1).

**Reliable detection of single template molecules by double PCR.** The double PCR overcomes the problem of limited amplification of a rare template sequence. Nested primers were used previously to amplify a single-copy human gene (20). During the first set of cycles, occasional interactions between the primers and mismatched sequences present in the genomic or carrier DNA led to some amplification of sequences other than the intended target sequence. The internally nested second primer pair ensured that only the desired sequences were further amplified during the second set of cycles. To take an example, in the amplification of the *gag* sequence the first step with the outer primers generated a 455-base-pair (bp) fragment. This product is then further amplified by using the inner primers to give a shorter (248-bp) fragment. Other nested outer and inner primer pairs were used in the same way. The procedure allowed the unequivocal identification of product HIV-specific DNA by agarose gel electrophoresis with ethidium bromide staining (Fig. 2).

Serial 10-fold dilutions of plasmid pBH10.R3 DNA were made in a solution of herring sperm DNA to obtain plasmid

DNA concentrations ranging from 65 ng/ml to 65 ag/ml. When 10- $\mu$ l samples from these dilutions, containing from 6.5 ng to 0.65 ag of plasmid, were amplified with the use of the outer *gag* primer pair, the lowest amount of starting material that gave a clearly visible band of the expected size (455 bp) was 65 fg (Fig. 2A). Addition of more Taq polymerase and further heat cycling did not measurably improve the sensitivity of the reaction (data not shown). On further amplification with the inner *gag* primers, the expected 248-bp band was detected from as little as 6.5 ag of starting material (Fig. 2B). The larger bands present in reactions which contained greater amounts of template were probably the result of copying DNA which was correctly amplified during the first reaction beyond the opposite internal primer site and on to the end of the template. Products of this type of reaction should accumulate linearly with cycle number rather than exponentially.

The mass of one copy of pBH10.R3, obtained by dividing the molecular weight of the 13-kbp double-stranded DNA molecule by Avogadro number, was 13 ag. Thus, the positive double PCR result with a nominal 6.5 ag of DNA (Fig. 2B) presumably represents a reaction which contained a single template molecule. If so, other samples taken from the same DNA dilution should contain no plasmid molecules. To test this, parallel replicate assays were carried out so that each reaction nominally contained 6.5 ag of pBH10.R3 DNA. The expectation was that plasmid molecules would be distributed to some but not all of the replicate reactions. In agreement with this expectation, some of the reactions gave a positive result while others gave a negative result (Fig. 2C). The positive reactions gave bands of similar intensity, and the proportion of positive to negative reactions was similar when the same set of assays was performed on separate occasions. The negative control reactions (with 1  $\mu$ g of herring sperm DNA) were invariably negative.

If the results were due to the distribution of single plasmid molecules to some but not all reaction tubes, the frequency of positive samples can be compared with an expected frequency calculated from the plasmid DNA concentrations. As a first approximation, an average (positive reactions/total reactions) could be taken. A more accurate method is to make allowance for reactions that contain two or more template molecules (which cannot be distinguished from those that contain one) by using the null class of the Poisson distribution. (The mean number of template molecules per reaction  $[m]$  is equal to  $-\ln[F]$ , where  $F$  is the fraction of negative reactions.) The corrected average may then be used to calculate the concentration of DNA molecules in the solution from which the samples were taken. An extensive series of reactions carried out with appropriate dilutions of pBH10.R3 DNA produced positive reactions at 78% of the frequency predicted (data not shown).

**Separate amplification of individual molecules from a mixture after dilution and distribution.** If the double PCR can detect single DNA molecules, then it should be possible to separate single molecules of two types from a mixture of the two by dilution and distribution and to amplify them separately. In order to test this proposition, a mixture was made of two clones derived from different HIV isolates, pBH10.R3 (HIV-HTLV-IIIB) and lambda HAT 3 (HIV-RF), and the mixture was diluted, distributed, and amplified as before by double PCR. The two variants differed in length by 42 bp in the sequence amplified by the *env* primers, so that the products could be readily distinguished by polyacrylamide gel electrophoresis (Fig. 3). The length of sequence between the *gag* primers is the same in each case. Figure 3, lanes 2

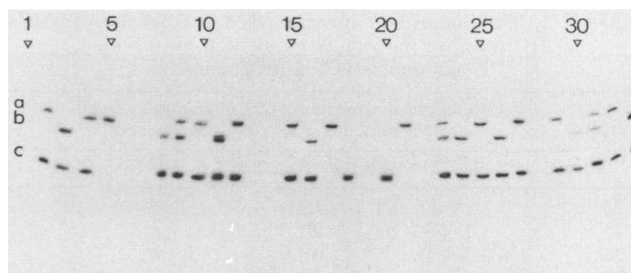


FIG. 3. Separate amplification of single molecules from a mixture. A mixture of pBH10.R3 and lambda HAT 3 was diluted in herring sperm DNA, and the appropriate dilutions were distributed to 28 tubes. Each sample contained (nominally) 6.5 ag of pBH10.R3 (0.5 molecules) and 60 ag of lambda HAT 3 (1.2 molecules). Another tube contained herring sperm DNA but no HIV sequences (negative control), while positive controls contained 65 ag of pBH10.R3 (5 molecules) or 400 ag of lambda HAT 3 (9 molecules), also in carrier herring sperm DNA. The first PCR contained the *env* and *gag* outer primers. A single sample of each PCR product was then amplified with radiolabeled inner *env* and *gag* primers. The products were run on an acrylamide gel, which was exposed to X-ray film. The amplified *env* sequences of lambda HAT 3 (a; 359 bp) and pBH10.R3 (b; 317 bp) are readily distinguishable; c (248 bp) is the amplified *gag* sequence. Lanes: 1, negative control (carrier DNA); 2 and 32, lambda HAT 3 alone; 3 and 33, pBH10.R3 alone; 4 to 31, the 28 samples distributed from the diluted mixture. Longer *gag* sequences are present in some samples (between bands marked b and c) for the reasons outlined in the legend to Fig. 2.

and 32 show the results of amplifying lambda HAT 3 alone, and lanes 3 and 33 show the results of amplifying pBH10.R3 alone. After dilution of the mixture of the two sequences (lanes 4 through 31), a clear separation of pBH10.R3 and lambda HAT 3 *env* sequences was seen. Of 28 reactions, 9 showed amplification of the pBH10.R3 *env* sequence (corrected mean, 0.39 molecules per reaction), while 13 of 28 reactions showed amplification of the lambda HAT 3 *env* sequence (corrected mean, 0.62 molecules per reaction). The number of reactions in which the amplification of both *env* sequences was expected to occur, calculated from these numbers with the assumption of independent distribution, was four. The observed number of three is in good agreement with expectation.

The results obtained with the *gag* and *env* primers were not in complete agreement. In 5 of the 28 replicates, either the *gag* or an *env* sequence was amplified, but not both. This is probably due to breakage of the molecules during the serial dilution. Note that the breakage of molecules did not affect the accuracy of the dilution method in estimating amounts of HIV provirus, except in the presumably rare event of breakage within the sequence to be amplified.

**Low levels of HIV provirus in circulating PBMCs of HIV-positive patients.** PBMC DNA was prepared from 52 blood samples obtained from 28 HIV antibody-positive patients and assayed in the double PCR reaction with *gag*, *pol*, and *env* primers (Fig. 1). In these screening experiments, 1- $\mu$ g samples of genomic DNA were used. All but one of the samples were positive for all three primer sets. A positive result was later obtained with the initially negative sample (RVL1; see below). Uniformly negative results were obtained with single PBMC DNA samples from each of 48 low-risk seronegative blood donors. Clearly negative results were also obtained on testing 5- and 10- $\mu$ g samples of negative blood donor DNA in the double PCR.

On the assumption that buffy coat cells are diploid (6.6 pg

TABLE 1. Titration of HIV proviral DNA in DNA from PBMCs of HIV-positive individuals by dilution, distribution, and double PCR amplification

Patient no.	(No. of positive reactions)/(no. of reactions tested) at a DNA dilution (in 10 <sup>3</sup> cell equivalents) of <sup>a</sup> :											
	500	100	50	25	13	10	5	2.5	2.0	1.0	0.5	0.25 0.13
74			1/1		1/1		1/1			5/13	1/3	0/3
75			1/1	4/6	2/8		0/1					
76		4/4	3/7	4/16			0/1					
77			1/1	2/2			4/4	7/11		0/5		
78	1/1			6/11			0/1					
79		5/5	1/1			3/3	8/9	1/4	1/3	3/11	0/1	
80		1/1	4/6	5/13			0/1					
82			2/2	2/2			5/5			4/5	8/17	
83			3/3	2/3		4/8	0/4					
84			3/3	3/3		4/8	6/12	0/5				
85							3/3		5/5	5/5	1/4	0/4
86						3/3	4/7	0/8				

<sup>a</sup> DNA was extracted from at least 10<sup>6</sup> cells in each case, and cell equivalence was calculated on the basis of a DNA content of 6.6 pg per cell. The dilutions shown were tested in the double PCR. Blank spaces signify that no tests were made at those dilutions.

per cell) and in the G1 phase of the mitotic cycle, 1 µg of DNA corresponded to 150,000 cells. In order to quantify the amount of provirus, 12 of the DNA samples were assayed by amplification after dilution and distribution (Table 1). These were a random sample to the extent that they were the first 12 fresh blood samples from different patients to become available. In each case, some of the selected dilutions gave both positive and negative reactions. The frequencies with which negative reactions occurred at appropriate dilutions were used to estimate the number of molecules of HIV-specific DNA in each sample. To allow comparison with results obtained by titrating cells (described below), amounts of DNA were expressed in cell equivalents (1,000 cell equivalents corresponds to 6.6 ng of DNA). In most cases, relatively few replicates were tested at the limiting dilutions, and the estimates are therefore subject to a degree of uncertainty. However, the amount of virus present in material from different patients clearly varies greatly, ranging from 12 copies per 10<sup>6</sup> cells (patient 76) to 1,360 copies per 10<sup>6</sup> cells (patient 82) (Table 2; reciprocals of values listed in column 2). There is a strong positive relationship between the success of virus isolation from a patient over the previous year and the amount of HIV provirus detected by the PCR (Table 2, columns 1 and 2); according to the Spearman coefficient of rank correlation, the probability of the observed association occurring by chance is <0.02. No relationship was evident between the number of provirus molecules and the proportion of either T4 cells or monocytes present (Table 2, columns 4 and 5). This discounts the possibility that the variation in the number of target cells accounts for the variation in the amount of provirus. Finally, a positive association existed between disease status and amount of provirus (Table 2, column 6); samples from patients with AIDS or AIDS-related complex (Centers for Disease Control [CDC] categories IVc and IVa) usually contained greater amounts of provirus than samples from asymptomatic patients, although this association was not statistically significant ( $P = 0.2$ ). Thus, the likely explanation of the reported association between isolation rate and disease state (2, 17, 27) is a dependence of both on the frequency of provirus.

The negative sample (RVL1; see above) was the only sample obtained from one of the patients. Three 5-µg por-

TABLE 2. Comparison of amount of HIV provirus with success of virus isolation and disease status

Patient no.	No. of cells per single provirus <sup>a</sup>	Success of isolation <sup>b</sup>	% CD4 <sup>+</sup> lymphocytes in PBMCs	% Monocytes in PBMCs	CDC stage (clinical status)
76	83,000	0/2	11	24	II <sup>c</sup>
80	50,000	ND <sup>d</sup>	17	9	II (weight loss)
75	30,000	0/6	29	20	IVa, IVc (renal tuberculosis)
78	30,000	0/1	20	33	II
83	14,000	0/2	24	14	II
84	10,000	0/3	17	22	II
86	5,800	ND	12	16	II
79	3,300	2/4	21	14	IVc (oral candida infection)
77	2,500	1/2	8	28	IVa (for 2 yrs; on 3'-azido-3'-deoxythymidine)
74	2,000	4/4	10	13	IVa, IVc (oral candida infection)
85	900	2/4	12	16	IVc (oral candida infection)
82	700	2/2	21	13	II

<sup>a</sup> Number of PBMCs calculated to contain a single molecule of provirus; derived from data shown in Table 1.

<sup>b</sup> Results obtained over the preceding year ([number of cultures which became positive for p24 antigen]/[number of cultures tested]).

<sup>c</sup> II, Asymptomatic.

<sup>d</sup> ND, Not determined.

tions of this sample were positive in the double PCR, and when 13 1-µg portions of this sample were assayed, 7 were positive (data not shown). Thus, all of the samples from seropositive patients that we have examined were positive either in the initial screen or on closer examination. The 7 of 13 positive samples indicate that the sample from patient RVL1 had five provirus copies per 10<sup>6</sup> cells.

**Low copy number of HIV provirus in infected cells.** Because the number of copies of HIV provirus present in a single infected PBMC in vivo is unknown, the titration of PBMC DNA from patient material does not provide a measure of the number of infected cells present. However, the number of infected cells can be estimated by diluting and distributing the cells themselves before DNA isolation. In this case a Poisson distribution of HIV-positive cells, rather than of DNA molecules, should occur at the limiting dilutions.

This cell dilution and distribution procedure was carried out with samples obtained from four patients. The DNA prepared from each of the distributed samples was amplified with the double PCR to score the samples which contained at least one HIV provirus. From these data, the proportion of cells which contained one or more provirus molecules was calculated (Table 3). The values obtained ranged from one HIV-positive cell per 16,700 cells (patient 84) to one HIV-positive cell per 500 cells (patient 85). This corresponds to one HIV-positive T4 cell per 2,800 (patient 84) and per 55 (patient 85) T4 cells. Similarly, if both T4 cells and monocytes are infected, the calculation gives one HIV-positive cell per 6,500 (patient 84) and per 130 (patient 85) infectable cells.

The number of provirus molecules per cell may be calculated without reference to the proportion of infectable cells from two measured values: (i) the proportion of all cells which contain provirus and (ii) the proportion of DNA cell equivalents which correspond to a single provirus molecule.



TABLE 3. Proportion of PBMCs from HIV-positive patients which contained HIV proviral DNA

Patient no.	Type of dilution	(No. of positive reactions)/(no. of reactions tested) at a dilution (in units of 10 <sup>3</sup> cells or cell equivalents) of:									No. of cells or cell equivalents per single molecule of HIV provirus
		13	10	5	2.5	2.0	1.0	0.5	0.25	0.13	
83	Cell	3/4	0/2								7,200
	DNA	4/8	0/4								14,500
84	Cell	2/2	0/2								16,700 <sup>a</sup>
	DNA	4/8	6/12	0/5							10,800
85	Cell	2/2	2/2	2/2	2/2	2/2	4/6				460
	DNA		3/3	3/3		5/5	5/5	1/4	0/4		870
86	Cell	3/6	2/4								10,800
	DNA		3/3	4/7	0/8						5,900

<sup>a</sup> Values for  $1.0 \times 10^4$  and  $1.3 \times 10^4$  cells were aggregated.

In general, the titration of cells and DNA gave very similar results, showing a similar prevalence of positive reactions at limiting dilution (Table 3). The similarity in the two sets of figures provides very strong evidence that in the patients tested there is approximately one copy of provirus per HIV-infected cell. The relatively small data sets do not justify a more precise interpretation than this. The efficiency of the method that was used to prepare DNA from the diluted cells is between 60 and 75% (see Materials and Methods). Correcting for this would only slightly increase the predicted numbers of infected cells.

**Nucleotide sequences of PCR products confirm the amplification of single molecules.** As a further confirmation that single molecules of proviral DNA from PBMC DNA were being detected in the double PCR, amplified DNA was recovered from positive reactions obtained at the limiting dilutions and sequenced. It is known that proviruses with different nucleotide sequences may be present in a single isolation (26). If so, and if the positive reactions were due to the amplification of several molecules, sequence ambiguities should be observed, with more than one base sometimes appearing at the same position. Furthermore, differences in sequence will in general not be observed between parallel PCR products from the same DNA sample. On the other hand, if the positive reactions were due to the amplification of single molecules, each should give an unambiguous sequence. Also, if more than one provirus sequence was present in a given blood sample, different sequences should be amplified in different parallel reactions.

Limiting-dilution PCR products from patients 75, 76, and 79, amplified with *gag* primers, were sequenced directly by using primer 883. In the case of patient 75, 13,000 cell equivalents of DNA were amplified, in the case of patient 76, 25,000 cell equivalents were amplified, and in the case of patient 79, 1,000 and 2,000 cell equivalents were amplified. These dilutions gave a low frequency of positive reactions (Table 1) and hence, if our conclusions are correct, the probability was high that single molecules would be amplified. Each amplification product gave an unambiguous readable sequence of at least 175 bases. Five of the seven sequences are unique, with variation both between samples derived from different patients and between parallel amplification reactions originating from the same DNA preparation (Table 4). The sequence differences showed a bias towards synonymous mutations, an indication of functional constraints on variation in this region consistent with that observed between published *gag* sequences (15). As an

TABLE 4. Variation between single provirus nucleotide sequences derived from HIV-infected patients<sup>a</sup>

Patient no.	Reaction no. <sup>b</sup>	Nucleotide sequence no.							
		36	69	136	165	171	183	184	201
75				C		G	T	T	G
76	a			C		G			
	b	A				G			
	c			C	T				
79	a		G		T				
	b								
	c		G		T				

<sup>a</sup> Deviations shown are from the consensus of the published sequences shown in Fig. 1. The position indicated relates to the 5' end of the fragment, i.e., the 5' end of the inner *gag* primer (Fig. 1, primer 883). Silent mutations are in boldface type.

<sup>b</sup> Positive reactions obtained after distributing a limiting dilution.

illustration of the results obtained, Fig. 4 shows a difference in sequence between two reactions (b and c) from the DNA of patient 79.

Finally, a positive amplification reaction obtained from 10<sup>5</sup> cell equivalents of DNA from patient 76 was sequenced. This larger amount of template DNA was likely to contain several copies of provirus, and this was confirmed by the discovery of two ambiguities in the sequence. At each of these sites two bands of similar intensity were seen rather than the expected single band. In summary, the absence of ambiguities and the observed variation between and within patient samples when limiting dilutions of PBMC DNA were

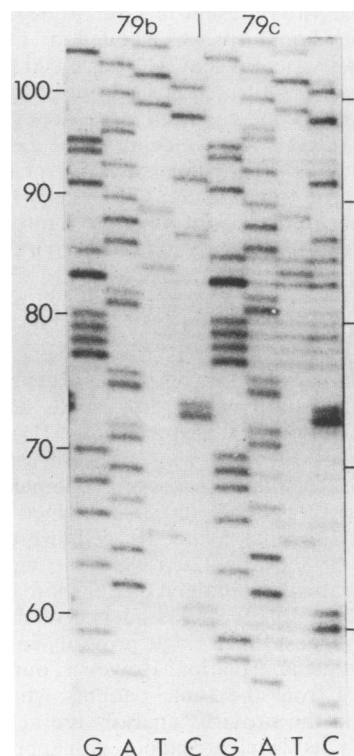


FIG. 4. Direct nucleotide sequencing of double PCR products. The PCR products were derived from single molecules of HIV provirus (b and c) from patient 79. The sequence shown lies between the inner *gag* primers (Fig. 1). A single nucleotide difference (A or G, position 69) can be seen.

amplified support the evidence cited above that the double PCR detected single molecules of HIV provirus.

## DISCUSSION

**Frequency of provirus-containing cells.** The PBMCs used in these experiments were donated during 1988 and 1989 by HIV-seropositive hemophiliacs who are believed to have been infected in 1984 (19). Seven were asymptomatic, and five displayed AIDS or AIDS-related complex (Table 2). The most striking feature of the results is the extremely low level of HIV provirus present in the circulating PBMCs in most cases. The samples that were then used to assess how many PBMCs carry provirus came from three asymptomatic patients and one with AIDS. The frequency of infected PBMCs from the patient with AIDS was about 1 per 500, while from the asymptomatic patients it was about 1 per 10,000 (Table 3). The fact that the relationship between circulating provirus and infected cells in each of these samples is close to one allows tentative extrapolation to the other eight samples. Overall, the frequencies ranged from about 1 per 700 to 1 per 80,000 cells, with a median value of 1 per about 8,000 cells (Table 2).

Of the seven asymptomatic (CDC stage II) patients, all but one fell into the range of 1 per 5,000 to 80,000 cells, while of the five CDC stage IV patients, all but one fell into the range of 1 per 700 to 5,000 cells. Thus, there is a positive association between the frequency of infected cells and disease status. The correlation with success of viral isolation is stronger, although this interpretation is tempered by the low number of isolation attempts made in some cases. Five patients who did not yield virus isolates in a total of 14 attempts all had virus present in 1 per 10,000 or more cells, while five from whom virus was isolated in 11 of a total of 16 attempts all had virus present in 1 per 3,000 or fewer cells. Schnittman et al. (29) titrated DNA from PBMCs from three patients with AIDS and reported a frequency of 1 provirus per around 100 CD4<sup>+</sup> positive cells. If one assumes that the CD4<sup>+</sup> lymphocytes are the sole reservoir of HIV provirus, then the proportion of infected CD4<sup>+</sup> cells in the patients with AIDS can be compared with the figures obtained by Schnittman et al. (29). Figures of one provirus per 100, one per 200, one per 700, and one per 8,700 CD4<sup>+</sup> cells were obtained in this way for the four patients with AIDS studied here.

The distribution of provirus frequencies among the 12 patients was similar to a distribution reported by Ulrich et al. (34). These authors attempted virus isolations from serially diluted PBMCs from 23 asymptomatic HIV-positive patients. A total of 10<sup>4</sup> cells was sufficient for a successful isolation in 65% of the 23 cases; for comparison, samples from 7 of our 12 patients (60%) contained one or more proviruses per 10<sup>4</sup> cells. Again, 10<sup>5</sup> cells were sufficient for an isolation in 83% of the 23 cases, while samples from all (100%) of our patients contained one or more proviruses per 80,000 cells. The close correspondence of the two sets of data seems to suggest that a single provirus-containing cell is sufficient to initiate an isolation. However, our own attempts to isolate virus from the same patients who donated the samples used in the provirus analysis are at variance with this. Our isolations were carried out with approximately 2 × 10<sup>6</sup> cells. Thus, all of our successful isolations occurred when at least 500 infected cells were present, and failures were invariably experienced with those samples which contained fewer infected cells (Table 2). The discrepancy may be more apparent than real. Ulrich et al. did not have

available numerical data on levels of provirus infection. Consequently, even though their patients were asymptomatic, it is possible that they represent a subset of such patients with high levels of provirus. Other uncertainties relate to the precise conditions under which virus was isolated in the two laboratories.

The direct comparison with isolation rates is of interest in relation to the numbers of PBMCs from patients with AIDS which were observed to contain transcriptionally active provirus (9). The more usual frequency was in the region of one per 10<sup>5</sup> cells, ranging up to one per 10<sup>4</sup> cells, while in 50% of the samples no cells containing viral RNA transcripts were observed. This must represent a frequency at least two orders of magnitude lower than that of provirus-containing cells in the patients with AIDS studied by us. Thus, it is possible that only a minority of infected cells are transcriptionally active and that only the same minority are capable of initiating an isolation. This view, which was previously proposed in part by Richman et al. (25), could presumably be tested by careful quantitation of provirally infected and actively transcribing cells within the same samples.

Two explanations of the low frequency of transcriptionally active cells may be considered. (i) The greater part of the provirus-containing cells are monocytes, which may exhibit lower rates of HIV transcription than lymphocytes (11, 21, 27). (ii) Most lymphocytes which contain provirus are transcriptionally silent or nearly so, a situation observed in vitro by Folks et al. (4). These explanations are not mutually exclusive. It should be possible to resolve the question fully by carrying out experiments similar to those reported here with sorted cell types.

If most lymphocytes which contain provirus are transcriptionally silent, it might be because the provirus that they carry is defective. We have some evidence bearing on this. Using the dilution, distribution, and double PCR method to amplify single sequences, we have determined a 180-bp sequence from the *gag* region of 19 single provirus molecules from six patients who were probably infected with the same virus (19) (7 of these are illustrated in Table 4). Among the sequences, there are 16 variable sites, i.e., sites at which one or more of the sequences differs from the consensus of all of them. None of these differences introduced either a stop codon or a frameshift (unpublished data). Similarly, analysis of a 170-bp sequence from the *env* region of 24 single molecules from four patients revealed 49 variable sites and a minimum of eight independent deletions or insertions. Again, no stop codons or frameshifts were found, and the deletions or insertions all involved a multiple of three nucleotides (P.S. and P.B., unpublished results). The total length of sequence considered here is approximately equivalent to one HIV genome. If the regions that were sequenced are typical, we may effectively exclude the possibility that most of the observed provirus is defective because of random mutational events.

These observations argue against the presence of a large proportion of inactive provirus in the PBMCs, but only with the assumption that the sites of inactivation are distributed through the genome at random. If instead they are confined to particular regions, such as the regulatory regions of the genome (*tat*, *rev*, *nef*, and the long terminal repeat), we would not have detected them in the course of this analysis. Also, gross disruptive rearrangements of the proviral DNA would usually not be detected by the PCR.

The possibility that much of the provirus population may be transcriptionally inactive is supported by the finding of latency after in vitro infection (4, 35). Even if only a small



proportion of ongoing virus production produces a latent infection *in vivo*, the proportion of latently infected cells may steadily increase as the infection progresses, since the cells infected with active virus are probably destroyed. Provirus may be integrated into the chromosome at a site at which its expression is prevented, or it may be transcriptionally inactive by virtue of being extrachromosomal. The question of proviral inactivation is currently under investigation.

**Sequence variation in HIV.** The analysis of nucleotide sequence variation in HIV has presented serious difficulties. It is difficult to isolate molecular clones of provirus directly from PBMCs because of the low ratio of provirus genomes to cellular genomes. Once virus has been isolated by *in vitro* propagation, which increases the proportion of infected cells, provirus may be more readily cloned. However, the *in vitro* propagation of virus introduces new uncertainties. Selection of variants which grow more successfully *in vitro* may occur, or inviable variants which are sustained by complementation or phenotypic mixing in multiply infected cells may accumulate. Thus, viral sequences isolated after *in vitro* propagation may not be truly representative of the viral sequences present in the donor.

Another approach is to amplify the proviral sequences which are present in DNA extracted from PBMCs by the PCR method and to isolate molecular clones of the amplified product for sequencing. This method is also liable to give misleading results. The Taq polymerase introduces an error in about 1 nucleotide in 9,000 (33). This means, for example, that after 20 cycles of amplification, 20% of 200-bp strands and 68% of 1-kilobase strands will carry one or more errors. (The proportion of strands with no errors is  $\{1 - (L/2E)\}^N$ , where  $L$  is the effective length of the template in base pairs,  $E$  is the reciprocal error rate per nucleotide, and  $N$  is the number of cycles.) Thus, it will be difficult or impossible to decide, after cloning and sequencing single amplified molecules, which differences are original and which were introduced by the polymerase. The dilution and distribution method which we have described here overcomes all of these difficulties. Only if an error occurs in the first cycle of amplification is it likely to cause a problem of interpretation. In this case, the error affects one-quarter of the fully amplified product; errors that occur during the second and subsequent cycles affect progressively less of the product. First-cycle errors will occur only rarely, during 1 in 10 amplifications of a 500-bp sequence or 1 in 25 amplifications of a 200-bp sequence. Such errors will be detected as ambiguities. The frequency of reactions which show a sequencing ambiguity because more than one template molecule is present will usually be greater than this. From the Poisson distribution, when 50% of reactions are positive, about one-third of the positive reactions will contain two template molecules; when 25% are positive, about one-eighth of the positive reactions will contain two templates. The best practice would seem to be to disregard all reactions in which sequence ambiguities arise.

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